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Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7

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ABSTRACT

Allyl isothiocyanate (AIT) is derived from the glucosinolate sinigrin found in plants of the family Brassicaceae. It is a well-recognized antimicrobial agent against a variety of organisms, including foodborne pathogens such as *Escherichia coli* O157:H7. The efficiency of this natural agent in reducing *E. coli* O157:H7 numbers in food products have been reported. However, few have examined the mechanism by which AIT, and perhaps most of the isothiocyanates, kill *E. coli* O157:H7. In the present report, AIT showed greater antimicrobial activity at low pH values. For example, at pH 4.5 and 5.5 the MIC was 25 μL/L, while at pH 8.5, 500 μL/L was required to inhibit bacterial growth. This mustard-derived compound exhibited a high decomposition rate in water at 37 °C. Its degradation profile contained 3 major products and of these, diallylthiourea represented the largest (~80%) component. The decomposition products did not show antimicrobial activity towards *E. coli* O157:H7, even when combined with a sub-lethal dose of AIT (10 μL/L). AIT may only be antimicrobial in its original form and any further degradation in water is undesirable. AIT interactions with thioredoxin reductase and acetate kinase were also subjects of this study. AIT at 10 to 100 μL/L was able to significantly inhibit both enzymes, but only 1 μL/L was needed to decrease the activity of thioredoxin reductase. From these results, it can be postulated that: 1) AIT is a more effective antimicrobial at low pH values and its degradation reduces this activity; 2) decomposition products in water might not participate in the antimicrobial action of AIT; and 3) AIT seems to have a multi-targeted mechanism of action, perhaps inhibiting several metabolic pathways and damaging cellular structures.

1. Introduction

Isothiocyanates naturally occur in plants of the family Brassicaceae. The enzyme myrosinase is released after injury or disruption of cell membrane or walls and catalyzes cleavage of the glucose molecule from glucosinolates, generating isothiocyanates, thiocyanates and nitriles (Delaquis and Mazza, 1995). Allyl isothiocyanate (AIT) is derived from the glucosinolate sinigrin and is responsible for the characteristic pungency found in horseradish and mustard pastes (Cejpek et al., 2000). Several researchers have described the antimicrobial properties of AIT against various foodborne pathogens (Isshiki et al., 1992; Lin et al., 1999; Rhee et al., 2003), but the specific mechanism of its microbiocidal activity is still unknown.

Zsolnai (1966) reported that thioglycollate and cysteine could diminish the antibacterial action of isothiocyanates and related this event to the possible reaction of the isothiocyanate with the thiol groups of these compounds. He hypothesized that the antimicrobial action of isothiocyanates may be linked to the inhibition of sulfhydryl-enzymes. Subsequently, there has only been a single report where the interaction of isothiocyanates with enzymatic systems has been verified. In that work, Kojima and Ogawa (1971) observed a reduction of oxygen uptake when allyl, methyl, phenyl and β-phenylethyl isothiocyanates were tested against 3 different species of yeast. The authors also showed significant enzymatic inhibition of cytochrome *c* oxidase by allyl isothiocyanate. However, the results of this work raise questions, since the levels used to achieve both enzymatic and oxygen uptake inhibition were 200 times greater than the actual minimum inhibitory concentration of the isothiocyanates for those organisms.

Reactions involving isothiocyanates also were studied by Kawakishi and Kaneko (1985, 1987), where they showed that allyl isothiocyanate was able to slowly cause disulfide bond oxidative cleavage in cystine residues and to react with the terminal amino groups of lysine and arginine. However, these authors did not relate these findings to the possible reaction of AIT with microbial proteins. In addition, the reactions were analyzed at temperatures over 37 °C with intensive stirring, which can facilitate AIT–protein interactions.

Lin et al. (2000) showed that AIT caused damage in the cell membrane of *Escherichia coli* K-12, leading to leakage of cellular metabolites. However, Ahn et al. (2001) found no leakage of ATP or damage in the cell wall when AIT was tested against *Listeria monocytogenes*, but internal levels of ATP were reduced. In addition,

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the authors observed that the AIT-treated cells had altered internal structure in comparison to non-treated bacteria when analyzed by transmission electron microscopy.

Degradation of allyl isothiocyanate in water has been reported (Chen and Ho, 1998; Kawakishi and Namiki, 1969; Ohta et al., 1995; Tsao et al., 2000), but no study relating this degradation to AIT antimicrobial activity has been published. Kawakishi and Namika (1969) demonstrated that AIT was degraded in aqueous solutions at 37 °C, forming a variety of by-products which produced a garlic-like odor. This decomposition was slower than with other isothiocyanates such as ρ-hydroxybenzyl isothiocyanate and produced negligible amounts of SCN^-. The latter authors also showed that AIT reacted with water and the main products of this reaction were identified as allyl dithiocarbamate, diallyl tetra- and penta-sulphide, sulfur and N,N′-diallylthiourea. In addition, the degradation process was dependent on temperature and pH, where AIT was more easily decomposed at higher temperatures and alkaline pH values (Tsao et al., 2000).

The purposes of the present study were to evaluate the antibacterial activity of AIT against *E. coli* O157:H7 at different pH values; to examine the participation of eventual degradation products in this activity; and to test AIT for inhibitory action against two enzymes important in the metabolism of *E. coli*: thioredoxin reductase, implicated in ribonucleotide synthesis; and acetate kinase, related to energy metabolism.

2. Material and methods

2.1. Chemicals

Allyl isothiocyanate was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA); diallylthiourea from Alfa Aesar (Karlsruhe, Germany); 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetyl phosphate, adenosine 5′-diphosphate sodium salt (ADP), adenosine 5′-triphosphate disodium salt (ATP), adenosine 5′-triphosphate (ATP) bioluminescent assay kit, bovine serum albumin (BSA), *E. coli* acetate kinase (AK), *E. coli* thioredoxin reductase (TR), ethylenediaminetetraacetic acid (EDTA), HEPES buffer, and reduced β-nicotinamide adenine dinucleotide 2′-phosphate tetrasodium salt (NADPH) were purchased from Sigma Chemical Co (St. Louis, MO, USA). The solvent components, methanol, acetonitrile and acetic acid, were HPLC grade (Fisher Scientific Co., Fair Lawn, NJ, USA). Other chemicals noted were of analytical grade.

2.2. Bacterial strains

Experiments involving bacterial growth used a five strain mixture of *E. coli* O157:H7. The strain LCDC 7283 (pathogenic, hamburger isolate) was provided by Dr. R. Khakria, Laboratory Centre for Disease Control, Ottawa, Canada. Strains 02-0628, 02-0627, 00-0351 and 02-0304 (non-pathogenic, human isolates) were supplied by Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency, Canadian Centre for Human and Animal Health, Winnipeg, MB, Canada. Each bacterial strain was transferred into fresh Luria-Bertani (LB) broth (Difco Laboratories, Sparks, MD, USA) and allowed to grow overnight (16 h) at 37 °C. Then, 50 μl of each strain was added together to a single tube containing 9.75 ml LB broth and cultured for another 16 h at 37 °C prior to the experiments. The organisms were cultured again and 0.1 ml samples at mid-exponential phase were used (optical density ~0.6, at 600 nm represented approximately 7 log CFU/ml). Cultures were handled in a sterilized laminar flow hood and all biological material was autoclaved at 121 °C for 20 min before disposal.

2.3. Determination of minimum inhibitory concentration at different pH values

Luria-Bertani broth was prepared following the manufacturer specifications and NaOH or HCl was used to reach the desired pH (4.5, 5.5, 6.5, 7.5, 8.5). Bacteria were grown overnight at each one of the experimental pH values for adaptation and were re-inoculated in fresh broth. Aliquots of 0.1 ml were taken from the culture mixture at mid-exponential phase (optical density ~0.6) and added to screw-capped tubes containing 9.9 ml of LB broth at the corresponding pH value. Concentrations of allyl isothiocyanate (AIT) ranging from 10 μL/L to 500 μL/L were used to determine the minimum inhibitory concentration at specific pH values. The tubes were incubated at 37 °C at a shaker speed of 200 rpm (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL, USA) for 48 h. Absence of growth (no increase in measured OD) was considered as the MIC.

2.4. Antimicrobial activity of degradation products

Allyl isothiocyanate at 500 μL/L was added to deionized water and stored 48 h at 37 °C. The AIT remaining was then extracted using hexane (adapted from Shahidi and Gabon, 1990) and the extracted aqueous phase (EAP) was used for identification analysis (HPLC and LC-MS) and tested against *E. coli* O157:H7. For the latter tests, 8.9 ml LB broth were added to screw-capped tubes, followed by 0.1 ml *E. coli* O157:H7 mixed inoculum (OD ~ 0.6). After the inoculum was added, the tubes were treated with one of the following solutions: 1 ml of the EAP, 1 ml EAP + AIT 10 μL/L, 1 ml of deionized water + AIT 10 μL/L or 1 ml of deionized water (control). Cell density was measured after 16 h (600 nm, Novaspec Plus spectrophotometer; Biochrom, Cambridge, England) at 37 °C. Diallylthiourea, diallylurea and allyl disulfide, the main degradation products found in the EAP, were also tested against the *E. coli* O157:H7 mixture. In this experiment, 4 mg diallylthiourea and 0.5 mg of diallylurea were dissolved in 10 μL ethanol in a microtube (1.5 ml, flat top microcentrifuge tubes; Fisher Scientific) and 990 μL of LB broth (stock solution) were added. Diallyl disulfide, the third decomposition product, also was added to the tube (0.5 μL) and the mixture (degradation products solution) was filter sterilized (25 mm syringe filter, 0.22 μm pore size, Fisher Scientific). To test tubes containing 9.7 ml of LB; 0.1 ml of *E. coli* O157:H7 mixed inoculum (OD ~ 0.6) and 0.1 ml of the degradation products solution were added. All groups tested received 0.1 ml of a 1 ml/L AIT solution (sub-lethal level of 10 ppm) or an equal amount of water to bring the final volume to 10 ml. Control groups were prepared with bacteria treated with 10 μL/L of AIT or bacteria with no AIT exposure. All tests above were incubated at 37 °C for 16 h and were repeated 3 times and each experiment was done in triplicate ($n = 9$).

2.5. HPLC and LC-MS apparatus and operating conditions

Analyses were conducted on an HPLC (Waters 2695, Waters Corp., Milford, MA, USA) system equipped with a photodiode array detector (Waters 996), Empower software, and autosampler (Waters 717 plus). The separation of the compounds was done at room temperature using a Symmetry C18 column (Waters Co., 4.6 × 250 mm i.d. 5 μm). Elution was carried out isocratically for 40 min at a flow rate of 0.5 mL/min, using a solvent system containing 40% (v/v) acetonitrile (0.1% v/v acetic acid) and 60% water (0.1% acetic acid). The injection volume used was 10 μl. A dual absorbance detector was used to simultaneously measure the absorbance at 220 and 254 nm in order to verify the presence of AIT and its degradation products.

LC-MS was used to identify the decomposition products of AIT in aqueous solution. LC separation was done on an ACQUITY™ UPLC system consisting of a binary pump, a sample manager, and a PDA detector set at 254 nm (Waters Corp.). An ACQUITY™ UPLC BEH C18 column 1.0 × 100 mm, i.d. 1.7 μm was used for detection of the reaction products with a flow rate of 0.2 ml/min. Five μL samples were injected into the LC and analyses were done isocratically in a solvent system composed of 60% water (solvent A) and 40% v/v acetonitrile (solvent B), both containing 0.1% v/v formic acid. The eluting stream from the LC was introduced into a Waters Quatro Micro™ API mass spectrometer (Waters Corp.) equipped with an ESI Multi-Mode Ionization probe. All